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ABSTRACT:

We have previously shown that effective anti-tumor autoimmunity can be induced if a tumor-cell challenge is given to mice undergoing homeostatic T-cell proliferation, a process dependent on signaling by self-peptide/MHC and trophic cytokines. We investigated whether this principle can be applied to mouse models of advanced breast carcinoma, and whether the anti-tumor response can be enhanced using selected T-cell subpopulations, cytokines and tumor-vaccines. The results indicated that (a) homeostatic T-cell proliferation consistently elicits anti-tumor responses; (b) irradiation is more effective than T-cell depletion by antibodies in inducing anti-tumor responses mediated by homeostatic T-cell proliferation; however, irradiation (and/or the resulting lymphopenic state) may facilitate metastasis dissemination; (c) the frequency of T regulatory (Treg) cells increases during homeostatic proliferation, particularly in the presence of a growing tumor; in vivo depletion of Treg cells enhances the anti-tumor effect of homeostatic T-cell proliferation on subcutaneous breast carcinoma; (d) gamma/delta T cells, a lymphocyte subpopulation with significant anti-tumor activity, can be induced to undergo homeostatic proliferation, and this requires depletion of both alpha/beta and gamma/delta T cell compartments and availability of either IL-7 or IL-15; (e) the anti-tumor response is diminished in aged mice, and this correlates with inefficient homeostatic T-cell proliferation; this defect can however be corrected by provision of the trophic cytokine IL-7; (f) IL-7 complexed with anti-IL-7 antibodies and/or IL-2 complexed with anti-IL-2 antibodies induce T cell proliferation in both lymphopenic and non-lymphopenic mice; (g) in non-lymphopenic mice, IL-7/antibody and particularly IL-2/antibody complexes induce cytotoxic effector functions in CD8 T cells, and inhibit tumor growth, metastasis and mortality in a model of breast carcinoma; (h) tumor cells at early apoptotic stages induce production of type I interferons by dendritic cell subsets, promote interferon-dependent cross-priming of specific T cells, and protect mice from subsequent tumor challenges if used as a vaccine.

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INTRODUCTION

Breast cancer remains the second most common cause of cancer death among women. Current efforts in cancer immunotherapy focus on inducing autoimmune responses against tumor-associated antigens that are primarily encoded by normal unmutated genes. Breaking tolerance for self-antigens, however, remains a major challenge. Recent studies showed that, under lymphopenic conditions, peripheral T cells undergo "acute homeostatic proliferation" to re-establish appropriate cell numbers. Since this process depends on T cell recognition of self-peptide/MHC antigens and is accompanied by acquisition of effector functions, we hypothesized a link between lymphopenia-induced homeostatic proliferation and autoimmunity (1). We also suggested that induction of homeostatic T-cell proliferation concurrently with a tumor cell challenge may be a way to preferentially expand and activate otherwise tolerant lymphocytes and, hence, elicit effective anti-tumor autoimmunity (2). Our initial experiments with melanoma cell-challenged lymphopenic mice infused with syngeneic polyclonal T cells indicated that this is indeed the case (3). In the experiments supported by the current award, we wished to extend this observation and determine whether this approach can be used to inhibit progression of established breast tumors. Specific aims included (a) to apply the principle of homeostatic T-cell expansion to inhibit tumor growth in models of advanced breast cancer; (b) to enhance the efficacy of the response by manipulating the composition of the infused T cells; and (c) to potentiate the anti-tumor effect by using T cell survival and proliferation promoting cytokines, and/or by enhancing tumor-antigen presentation with efficient tumor vaccines.

BODY

The results summarized below describe experiments conducted over the entire research period and associated with tasks outlined in the approved Statement of Work. Note that the transplanted 4T1 breast carcinoma model has been found to appropriately recapitulate tumor growth and metastasis dissemination. Because of its convenience and fast kinetics, this model has been used throughout the project to evaluate and optimize therapeutic approaches based on T cell homeostatic expansion. Therefore, experiments with the spontaneous model constituted by FVB/neu-N transgenic mice (Tasks 1c, 2d, 3c, 3e), which would have required longer (and more expensive) experimentation protocols, have not been performed. Instead, more experiments have focused on the effects of enhancing T cell function with specific trophic cytokines, which appears a more relevant and promising approach for future translational studies.

Task 1.a. Define a protocol to induce lymphopenia using T cell-depleting antibodies for optimal homeostatic T-cell proliferation (accomplished in year 1)

In our initial studies, lymphopenia was induced before tumor challenge by sublethal irradiation (3). Because irradiation would affect tumor growth, assessing the effect of homeostatic T cell proliferation on established tumors would require induction of lymphopenia by other means than irradiation. Therefore, we evaluated the use of T cell-depleting antibodies (Abs) as an alternative way of lymphopenia induction. C57BL/6 (B6) mice (15/group) were treated with either sublethal irradiation (600 rads, control) or T cell-depleting Abs. We used anti-CD3 (145-2C11, 500 μg i.p.), anti-Thy1.2 (30-H12, 500 μg i.p.) or a combination of anti-CD4 (GK1.5, 200 μg i.p.) plus anti-CD8 (YTS169.4.2 200 μg i.p.) Abs, as described (4, 5). At days 1, 2, 3, 4 and 10 after treatment, 3 mice per group were sacrificed and cells in LNs and spleen enumerated. As shown in **Fig. 1**, both anti-CD3 and anti-Thy1.2 Abs efficiently depleted $CD4^+$ and $CD8^+$ T cell subsets, in both LNs and spleen. In contrast, depletion with anti-CD4 plus anti-CD8 was not as efficient (data not shown). Additionally, whereas anti-Thy1.2 did not cause obvious adverse effects to the mice, anti-CD3 treatment caused a toxic effect resembling that induced by bacterial superantigens. For both anti-CD3 and anti-Thy1.2, optimal depletion comparable to that observed 1 day after sublethal irradiation was achieved 4 days after injection. In a second experiment, B6 mice were similarly treated and transfused with 5×10^6 CFSE-labeled B6.PL ($Thy1.1^+$) LN cells. Transfusion was performed at day 1 after irradiation and at day 4 after Ab injection. At day 7

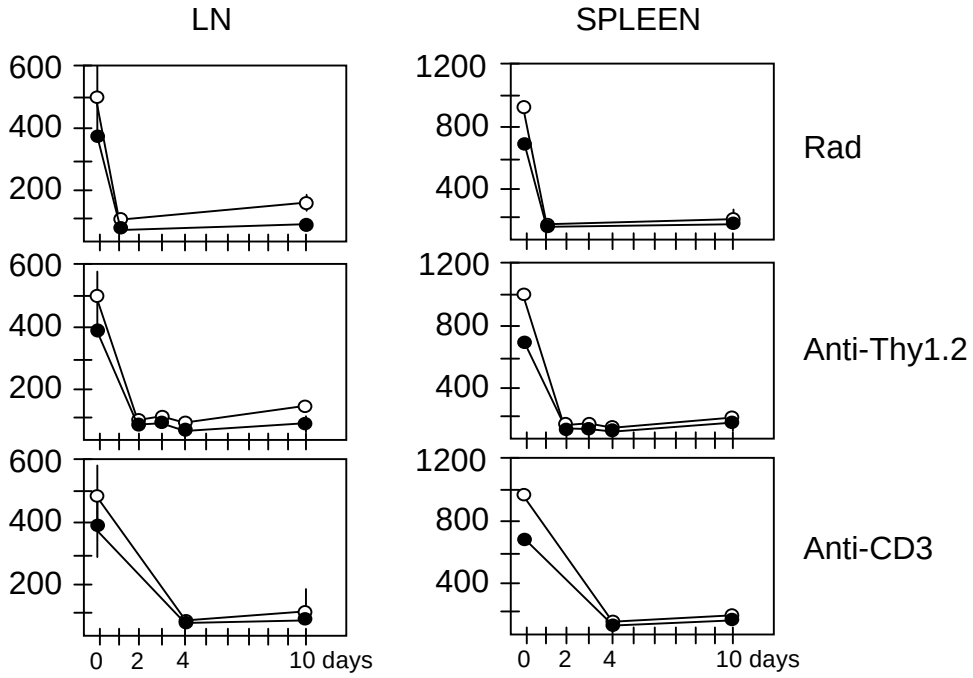


Figure 1. Efficient T cell depletion is achieved with anti-Thy1.2 and anti-CD3 antibodies. *B6 mice were treated (on day 0) with sublethal irradiation (600 rads), anti-Thy1.2 antibodies (500 μg i.p.) or anti-CD3 antibodies (500 μg i.p.). At the indicated days after treatment, mice were sacrificed, and LN and spleen CD4 (open circles) and CD8 (filled circles) T cells enumerated. Similar experiments were performed with mice treated with anti-CD4 and anti-CD8 antibodies (not depicted). Data represent T cell numbers × 10(-4) ± SD.*

post-transfer, 3 mice/group were sacrificed, and LN and spleen cells analyzed by FACS. As shown in **Fig. 2A**, homeostatic expansion of $CD4^+$ and $CD8^+$ T cells in mice that were T cell-depleted by anti-Thy1.2 and anti-CD3 was at least as efficient as that induced by sublethal irradiation. Recovery of donor cells in anti-Thy1.2-treated mice was also very efficient (**Fig. 2B**), whereas recovery in anti-CD3-treated mice was significantly reduced (<10%) compared to irradiated mice.

Task 1.b. Evaluate the efficacy of homeostatic T-cell expansion on established tumors of increasing size using an ectopic model of breast cancer (accomplished in years 1 and 2)

We compared the anti-tumor responses elicited by HTP in Ab-depleted *versus* irradiated mice. BALB/c mice ($Thy1.2^+$) were rendered (or not) lymphopenic by either anti-Thy1.2 Abs (day –4) or irradiation (day –1), then challenged subcutaneously (s.c.) with 10^5 4T1 breast carcinoma cells (day 0), and transfused (or not) with 50×10^6 syngeneic $Thy1.1^+$ LN cells (day +1). The results confirmed our previous study that HTP triggered by irradiation-induced lymphopenia leads to significant anti-tumor responses (**Fig 3A**). Thus, s.c. tumor growth was inhibited in irradiated mice undergoing HTP compared to controls. In addition, lung metastatic nodules and weights were reduced, and a few mice survived longer time in the group of irradiated mice in which HTP was enhanced by LN cell transfusion, compared to irradiated but non-transfused hosts. Surprisingly, however, lung metastases and mortality were increased in all irradiated mice (**Fig 3A**). Thus, in addition to induce HTP and anti-tumor autoimmunity, irradiation may also eliminate cell populations that may constrain metastasis spread particularly during the early phases of lymphocyte recovery. Alternatively, irradiation may enhance metastasis by altering the tissue microenviroment, e.g. through induction of cell apoptosis, activation of certain tissue-specific cellular components or release of cytokines, chemokines, MMPs and other mediators, as observed in some cancer-promoting inflammatory conditions (6-10).

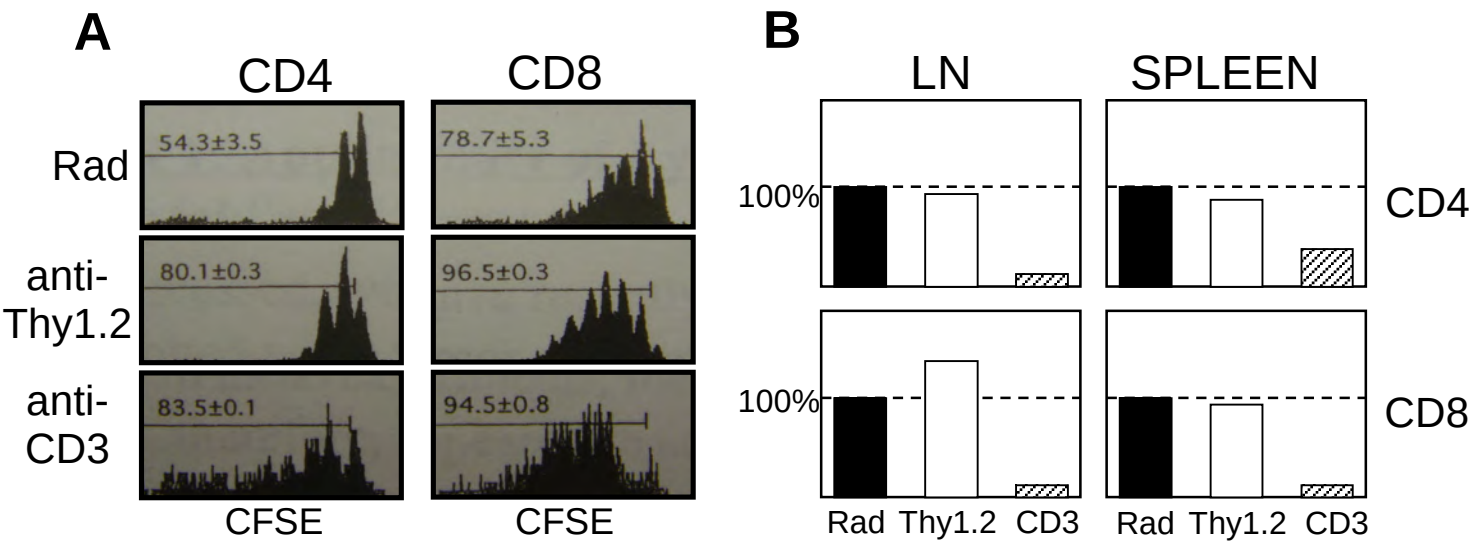
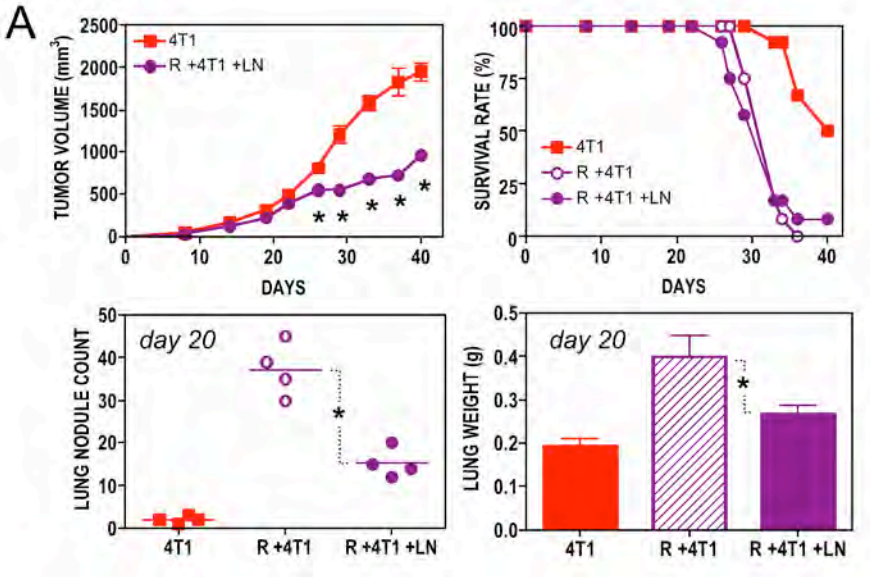


Figure 2. Efficient homeostatic T cell proliferation in recipient mice rendered lymphopenic with anti-Thy1.2 antibodies. *B6 mice were treated with sublethal irradiation (600 rads), anti-Thy1.2 antibodies (500 μg i.p.) or anti-CD3 antibodies (500 μg i.p.). At day 1 after irradiation, or at day 4 after antibody treatment, mice were transfused with 5 × 10(6) LN cells from Thy1.1(+) B6.PL mice. At day 7 after transfer, mice were sacrificed and LN and spleen cells enumerated and analyzed by FACS. (A) CFSE profiles on gated CD4(+) or CD8(+) T cells isolated from LN. Similar results were obtained by analyzing spleen cells. (B) Recovery of donor cells at day 7 post transfer expressed as a percentage (± SD) of cells recovered in sublethally irradiated control mice.*

Another surprising result was that HTP triggered by Ab-mediated T cell depletion was not associated with significant anti-tumor effects (**Fig 3B**). The reasons for this result are unclear, but it is possible that T cell-depleting Abs exert their function over a longer time, leading to delayed recovery of the endogenous T cell pool. In fact, additional studies showed that the number of endogenous T cells at day 20 after tumor challenge was > 2-fold reduced in Ab-treated, compared to irradiated, mice. Thus, experiments with reduced amounts of Abs may lead to better results. Another possibility is that, unlike Ab-treatment, irradiation depletes most immune cell subsets, some of which may play essential roles in containing proper T cell proliferation and/or activation. One of these cell populations may be constituted



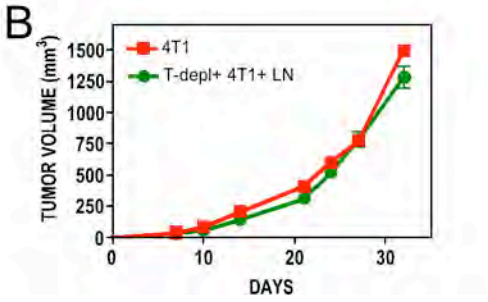


Figure 3. Irradiation is more effective than T-cell depletion in inducing HTP-mediated anti-tumor responses. *(A) BALB/c mice were irradiated (R), challenged with breast carcinoma 4T1 cells, and infused with LN cells. (B) BALB/c mice were treated with anti-Thy1.2 antibodies, then challenged and infused as in A. * , p<0.05 in t-test.*

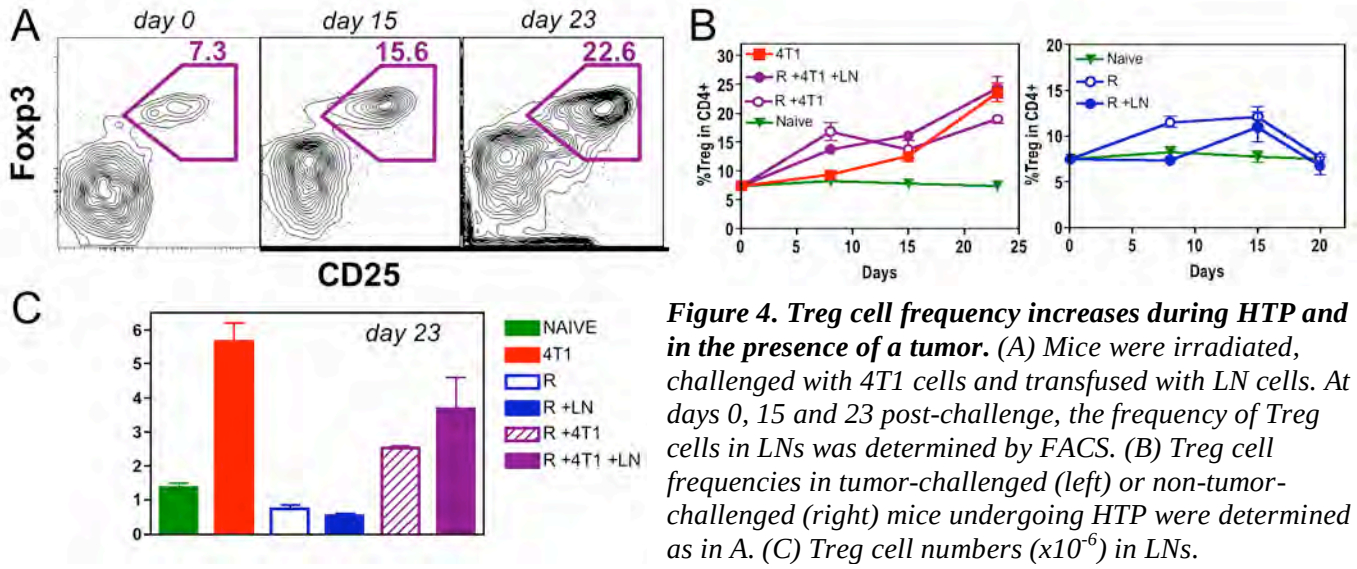


Figure 4. Treg cell frequency increases during HTP and in the presence of a tumor. *(A) Mice were irradiated, challenged with 4T1 cells and transfused with LN cells. At days 0, 15 and 23 post-challenge, the frequency of Treg cells in LNs was determined by FACS. (B) Treg cell frequencies in tumor-challenged (left) or non-tumor-challenged (right) mice undergoing HTP were determined as in A. (C) Treg cell numbers (x10^{-6}) in LNs.*

by NK cells, as their depletion was necessary to improve the anti-tumor effect of adoptively transferred T cell clones combined with vaccination and IL-2 treatment (11). Therefore, the use of additional Abs to deplete other relevant lymphocyte populations may improve the effect of T cell depletion.

Overall, the results indicate that irradiation is more efficient than Ab-mediated T cell depletion in inducing HTP-associated anti-tumor responses, as determined by the inhibition of s.c. tumor growth. However, lymphopenia may actually facilitate metastases dissemination. Thus, ameliorating the efficiency of T cell activation and expansion during HTP may lead to significant reduction in both s.c. tumors and metastasis. In the original proposal, we suggested that such amelioration could be achieved by various approaches, including by manipulating the composition of the expanding T cell population (e.g. depleting regulatory/suppressor T cells), by providing enhanced signaling from homeostasis related cytokines (e.g. IL-7), and by increasing the efficacy of tumor antigen presentation (e.g. using dendritic cell-based vaccines). The experiments described below were designed to address these possibilities.

Task 2.a. Determine whether depletion of regulatory/suppressor T cells from the infused cell population enhances the anti-tumor response in the ectopic model

Among T cell subsets with regulatory/suppressor activity, CD4$^+$CD25$^+$ T regulatory (Treg) cells are the best characterized (12, 13). These cells constitutively express the transcription factor Foxp3 and inhibit

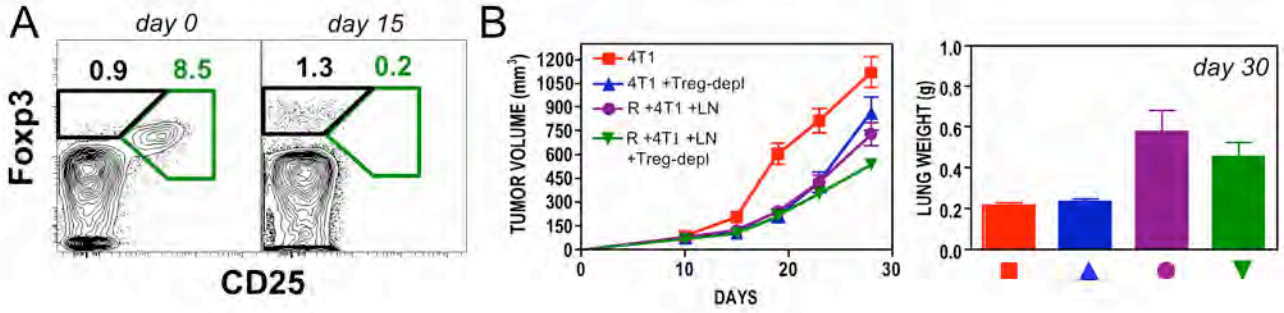


Figure 5. Treg cell-depletion improves the anti-tumor response mediated by HTP. *Mice were irradiated (or not), challenged s.c. with 4T1 breast carcinoma cells, infused (or not) with LN cells, and treated with Treg cell-depleting anti-CD25 antibodies. (A) On days 0 and 15 the frequency of Treg cells in the anti-CD25-treated mice was determined by FACS. (B) Volumes of s.c. tumors (left) at various time points and lung weight reflecting metastasis dissemination at day 30 were determined.*

activation and expansion of antigen-engaged T cells. Accumulating evidence indicates that Treg cells not only contribute to the maintenance of immunologic self-tolerance, but also inhibit both immunologic surveillance against autologous tumors and effective tumor-specific vaccination (12-15) (16-18). In addition, Treg cells expand during HTP (19), inhibit HTP of conventional T cell subsets (20), are induced during antigen-mediated T cell activation (21) and accumulate in cancer patients and tumor bearing mice (13, 22, 23). Thus, Treg-depletion, either from the infused LN cells or directly in tumor-challenged mice, may increase the HTP-mediated anti-tumor response.

To determine how the frequency of Treg cells is modulated during HTP in the presence or absence of a growing tumor, the following experiment was conducted. Mice were irradiated (or not), challenged with 4T1 cells and transfused (or not) with syngeneic LN cells. At days 7, 15 and 23 post-challenge, 3 mice per group were sacrificed and LN and spleen cells examined by FACS for the presence of $CD4^+CD25^+Foxp3^+$ Treg cells. As shown in **Fig 4A** and **4B**, in unmanipulated BALB/c mice Treg cells consistently accounted for ~8% of CD4 T cells. In contrast, in tumor-challenged mice Treg cells gradually increased, reaching ~22% by day 23. As the increase appeared accelerated in mice undergoing HTP, the effect of HTP was also examined in tumor-free mice. Indeed, Treg cell frequency was transiently raised during HTP, reaching ~12% by day 15, then returning to normal levels by day 20 after irradiation (**Fig 4B**). Cell enumeration also indicated that both tumor and lymphopenia-induced HTP contributed to the increase in Treg cellularity (**Fig 4C**). Moreover, in transfused mice, >50% of the Treg cells were of host origin, probably reemerging from the thymus after lymphopenia induction.

These data suggest that optimal inhibition of Treg cells would be achieved using depleting Abs *in vivo*, rather than by depleting the donor cells before injection. Therefore, mice were irradiated (or not), challenged with 4T1 tumor cells, and injected (or not) with LN cells. In addition, mice were injected (or not) every 5 days with the anti-CD25 monoclonal Ab PC61 to deplete Treg cells as described (14, 15). The results indicated that Treg cell-depletion was efficient, although a residual population of $Foxp3^+CD25^-$ cells remained detectable (**Fig 5A**). As previously reported, Treg-depletion significantly inhibited s.c. tumor growth in non-lymphopenic mice (**Fig 5B**). Treg-depletion also enhanced the effect of HTP on s.c. tumors, although lung metastases were only marginally affected.

Task 2.b. Determine whether enrichment of TCRgamma/delta T cells in the infused cell population enhances the anti-tumor response in the ectopic model (accomplished in year 1)

T cells expressing γδ T cell receptor (TCR) constitute a significant fraction of lymphocytes in peripheral lymphoid organs and blood and dominate in mucosa and epithelia of various tissues. Considerable evidence indicates that γδ T cells exhibit significant anti-tumor activities (24-26). We hypothesized that the principle of homeostatic expansion could be used to selectively enrich and prime this T cell subpopulation, so as to more efficiently exploit the associated anti-tumor effects. To explore this possibility, we initiated experiments to determine whether γδ T cells also undergo lymphopenia-induced proliferation and to characterize the homeostatic requirements of such process. The results of this study have already been published (27).

γδ T cells were isolated by negative selection (to avoid activation) from LNs and spleen of unmanipulated B6 mice, labeled with CFSE, and injected into syngeneic recipients. No proliferation was observed in non-lymphopenic recipients, suggesting competition with other lymphocyte populations. In contrast, homeostatic expansion of γδ T cells was detected in lymphopenic hosts. As previously observed with αβ T cells, γδ T cells also showed phenotypic changes during homeostatic proliferation, most notably upregulation of CD44 and downregulation of CD62L.

To identify the lymphocyte subsets that compete with γδ T cells during homeostatic proliferation, adoptive transfers were performed into mutant recipients that selectively lack specific cell populations. As expected, γδ cells proliferated in $RAG^{-/-}$ mice lacking both αβ and γδ T cells. Surprisingly, however, no proliferation occurred in non-irradiated $TCR\delta^{-/-}$ mice lacking γδ T cells, suggesting competition with αβ T cells. To investigate the mechanisms underlying such competition, we evaluated the involvement of MHC/peptide ligands, IL-7 and IL-15, i.e., the main modulators of αβ T cell homeostasis. Consistent with

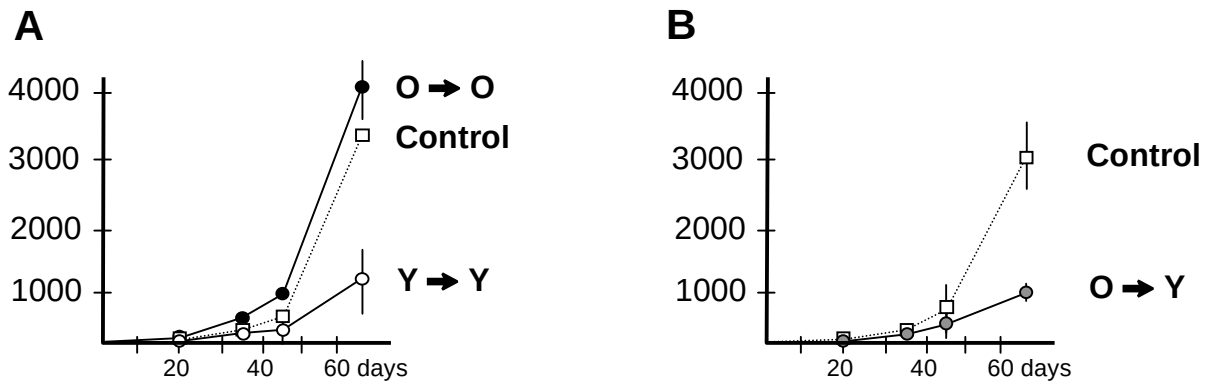


Figure 6. The anti-tumor effect of homeostatic T-cell proliferation is impaired in aged mice. *(A) Young (Y, 4 wks) and old (O, >80 wks) mice were sublethally irradiated, challenged s.c. with 5 × 10(5) B78D14 tumor cells (t = 0), and transfused i.v. with 5 × 10(6) total LN cells from age-matched donors. (B) Young (Y, 4 wks) mice were sublethally irradiated, tumor-challenged, and transfused with LN cells from old (O, >80 wks) donors. In both experiments, control mice were non-lymphopenic and tumor-challenged. Data represent tumor volume in mm(3) ± SD.*

the previous observation that γδ T cell populations are not decreased in MHC-deficient mice, we found normal homeostatic proliferation in $\beta_2M^{-/-}$ and I-Aβ$^{-/-}$ recipients. On the other hand, lack of homeostatic proliferation in double-deficient IL-7$^{-/-}$IL-15$^{-/-}$ mice, as opposed to normal expansion in single-deficient (IL-7$^{-/-}$ or IL-15$^{-/-}$) mice and in IL-7$^{-/-}$IL-15$^{-/-}$ mice treated with recombinant IL-7, suggested that γδ T cell homeostatic expansion requires either IL-7 (like most αβ T cells) or IL-15 (like memory CD8$^+$ αβ T cells).

If IL-7 and IL-15 were the only factors controlling γδ T cell homeostasis, γδ T cells would be expected to expand in non-irradiated TCRα$^{-/-}$ recipients lacking αβ T cells. Availability of IL-7 and IL-15 in these mice was demonstrated by the fact that adoptively transferred αβ T cells extensively proliferated, at rates similar as in RAG-1$^{-/-}$ mice . Remarkably, however, no homeostatic expansion was observed for γδ T cells in non-irradiated TCRα$^{-/-}$ mice. Because γδ T cells proliferated in RAG-1$^{-/-}$ hosts, these results indicate that, in addition to αβ T cells, γδ T cells also restrain acute homeostatic expansion of γδ T cells. Thus, it appears that the size of the γδ T cell pool in lymphoid organs is defined by availability of cytokines commonly used by other lymphoid cells (i.e., IL-7 and IL-15) as well as by additional γδ T cell-specific factors or ligands. Overall, the results indicate that lymphopenia-induced homeostatic proliferation in the presence of IL-7 and/or IL-15 may be used to expand and activate γδ T cells, and may allow to better exploit the anti-tumor potential of this lymphocyte subset.

Task 3.a. Define whether trophic cytokines enhance T cell homeostatic survival and proliferation in young and aged mice (accomplished in year 1)

Because the incidence of breast cancer increases in women with advanced age, and aging is associated with defective immune functions and decreased production of trophic cytokines, we examined whether the anti-tumor effect of homeostatic T-cell proliferation is impaired in aged mice. Young (4 wks of age) and old (>80 wks of age) B6 mice were sublethally irradiated to induce lymphopenia, challenged s.c. with 5×10^5 B78D14 tumor cells and transfused i.v. with 5×10^6 LN cells from age-matched syngeneic donors. A control group included tumor-challenged non-lymphopenic young mice. As shown in **Fig. 6A**, compared to young mice, tumor growth was not significantly inhibited in old mice. To determine whether the reduced anti-tumor response in aged mice was due to defects in the responding T cells or to age-related changes in the environment that supports such responses, additional groups were set in which young mice were rendered lymphopenic, tumor-challenged and transfused with LN cells from aged donors. The results indicated that impaired anti-tumor effects in the aged were not due to defective T cell responses, but rather

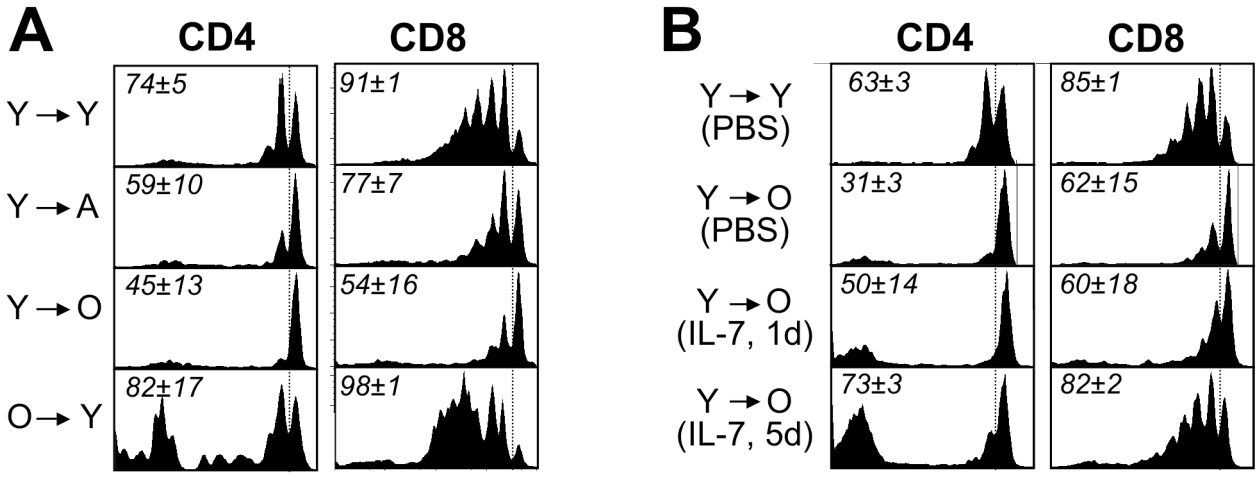


Figure 7. Impaired homeostatic proliferation in aging and correction by IL-7. *(A) CFSE-stained LN T cells (5 x 10^6) from young (Y, 4 wks) B6.PL mice were transferred into irradiated (600 rad) young (Y, 4 wks), adult (A, 48 wks) or old (O, 80 wks) B6 mice. Similarly, CFSE-stained LN T cells from old B6 mice (80 wks) were injected into young (4 wks) B6.PL mice (n = 3/group). CFSE profiles of donor (Thy1.2$^+$ or Thy1.1$^+$) CD4$^+$ and CD8$^+$ LN T cells were determined 7 days after transfer. Percentages of dividing cells are indicated. (B) CFSE-stained LN T cells (5 x 10^6) from young (Y, 4 wks) B6.PL mice were transferred into irradiated (600 rad) young (Y, 4 wks), or old (O, 108 wks) B6 mice treated s.c. with either PBS, IL-7 (1μg) for 1 day, or IL-7 for 5 days (n = 4/group). CFSE profiles were obtained at day 7 after transfer.*

to changes in the recipients, as T cells from old donors efficiently induced anti-tumor immunity when infused into lymphopenic young recipients (**Fig. 6B**).

Since aging is associated with considerable declines in the production of several cytokines, including the homeostasis-controlling IL-7 (28-31), we examined whether abnormal anti-tumor responses in aging correlate with reduced T cell homeostatic proliferation kinetics. CFSE-stained LN cells were transferred between allelically-different B6 and B6.PL mice in various age combinations (**Fig. 7A**). Indeed, the results indicated that aging is associated with impairment of homeostatic T cell proliferation. This abnormality appears not to be caused by a primary T cell defect but rather by changes in the microenvironment, since adoptively transferred T cells from aged donors displayed normal proliferation in lymphopenic young recipients.

To determine whether impaired homeostatic T cell proliferation in the aged can be corrected by supplementing cytokines, we treated old sublethally-irradiated recipients s.c. with 1μg recombinant mouse IL-7, either once (before transfusion) or 5 times (once before transfusion, then once a day for 4 days). Analysis at day 7 revealed that IL-7 given for 5 days significantly increased the frequency of proliferating T cells in aged recipients (virtually to levels registered in young recipients), whereas a single treatment had only marginal effects (**Fig. 7B**).

Task 3.b. Determine whether homeostasis-regulating cytokines enhance the anti-tumor response in the ectopic model (accomplished in years 2 and 3)

The above results suggested that, at least in aged individuals with deficient cytokine levels, anti-tumor responses could be enhanced with IL-7 and perhaps with other homeostasis-regulating cytokines. A very recent study, showed that the effect of IL-2 on HTP could be dramatically enhanced in young mice if IL-2 was complexed with certain anti-IL-2 Abs before injection (32). On this basis, we initiated experiments in collaboration with these investigators to examine whether this principle was also applicable to IL-7, i.e. whether IL-7/Ab complexes could be used to increase the efficiency of HTP. As

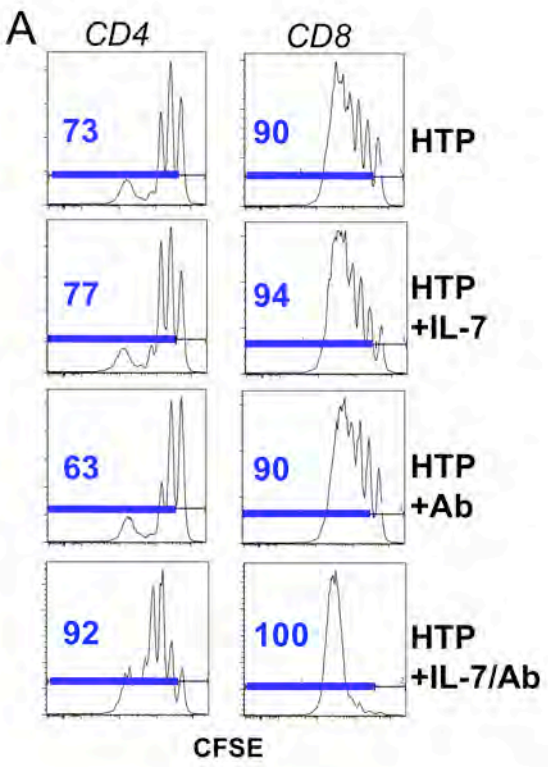


Figure 8. IL-7/Ab complexes enhance homeostatic T cell proliferation (HTP). *(A) BALB/c mice (Thy1.2) were irradiated on day -1, transfused with 50 x 10^6 CFSE-stained LN cells (Thy1.1) on day 0, and injected with IL-7, anti-IL-7 antibodies (Ab) or IL-7/Ab complexes on days 1, 4 and 7. At day 10, CFSE profiles in LNs were determined by FACS by gating on donor (Thy1.1) CD4 or CD8 T cells. (B) Total LN T cell numbers were determined in the mice used in the experiments described in A.*

we have previously observed, unlike the situation with aged individuals, administration of IL-7 alone did not enhance HTP in young mice, and the same was found for anti-IL-7 Abs (**Fig 8A**). In contrast, a significant acceleration of HTP was observed in mice injected with IL-7/Ab complexes, with the fraction of cells that proliferated within 10 days increasing from 73 to 92% for the CD4 subset and from 90 to 100% for the CD8 subset (**Fig 8A**). In addition IL-7/Ab complexes, but not IL-7 or Ab, induced a >2-fold increase in T cell numbers in both LN and spleen (**Fig 8B**).

Encouraged by these results, we examined whether acceleration of HTP by IL-7/Ab complexes was associated with more effective anti-tumor responses. Mice were irradiated (or not), challenged with 4T1 tumor cells, transfused (or not) with 50 × 10^6 LN cells and injected (or not) with IL-7/Ab complexes every 3 days for 2 weeks, and then once a week for the rest of the experiment. As shown in **Fig 9**, IL-7/Ab complexes reduced lung metastasis (p = 0.027 in Student t-test for HTP+IL-7/Ab compared to HTP) when examined at day 22 post-challenge, but had a limited effect on s.c. tumor

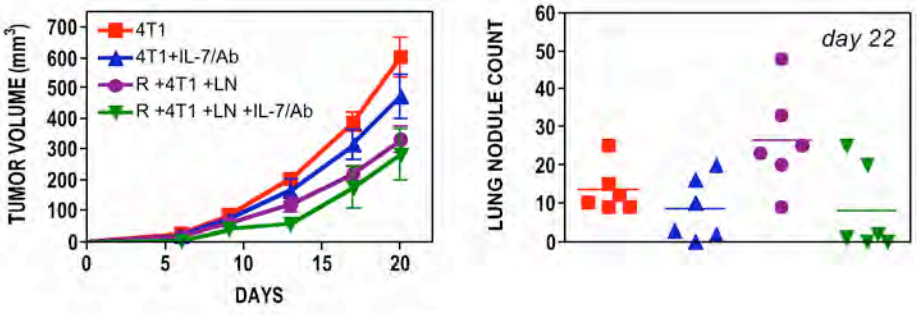


Figure 9. IL-7/Ab complexes enhance the anti-tumor response mediated by HTP. *BALB/c mice were irradiated (or not), challenged with breast carcinoma 4T1 cells, transfused (or not) with 50 x 10^6 LN cells, and treated (or not) with IL-7/Ab complexes. Growth of s.c. tumors was followed up to day 20. On day 22, mice were sacrificed and lung metastatic nodules counted under dissection microscope.*

growth and survival. Surprisingly, however, IL-7/Ab significantly extended survival of non-HTP control mice (not shown).

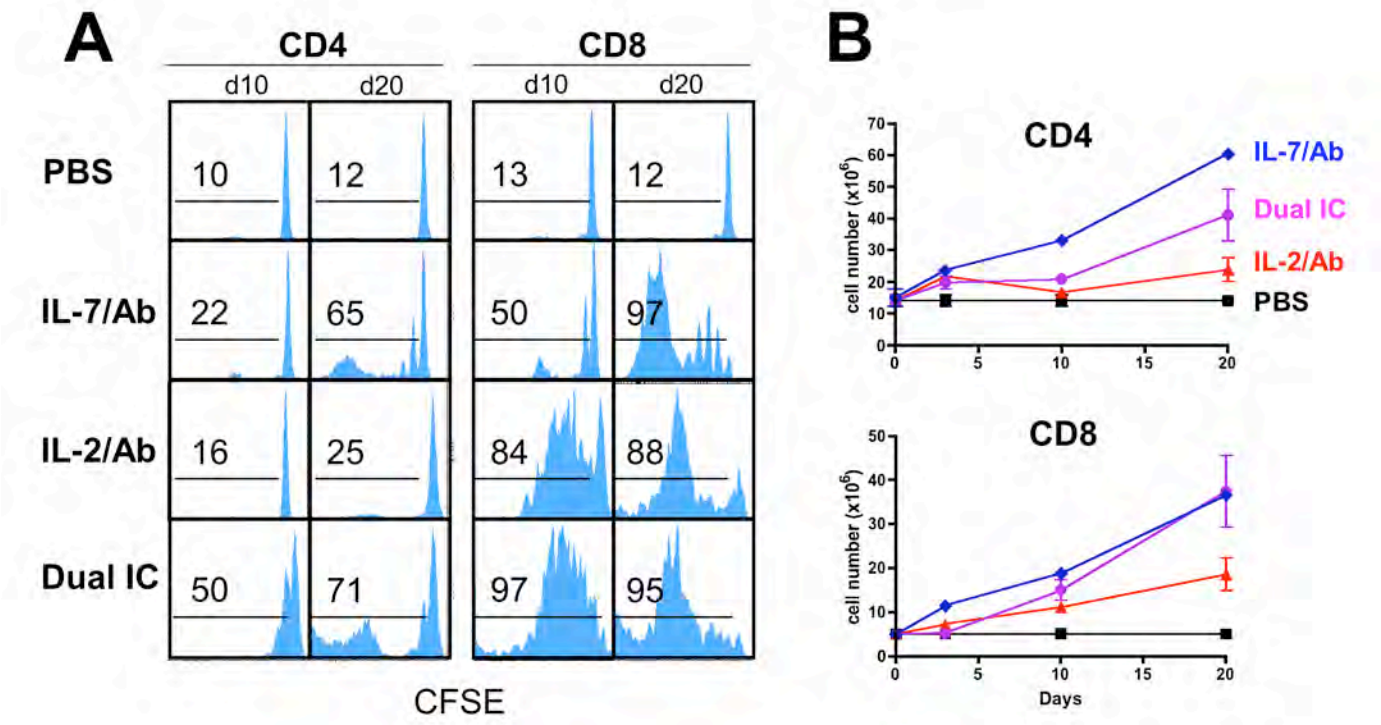


Figure 10. Cytokine/Ab complexes induce T cell proliferation in non-lymphopenic mice. *(A) BALB/c mice (Thy1.2) were transfused with 50 x 10^6 CFSE-stained LN cells (Thy1.1) on day 0, and injected with IL-7/Ab, IL-2/Ab or both immune complexes (dual IC) every 3 days. On days 3 (not depicted), 10 and 20, CFSE profiles in LNs were determined by FACS by gating on donor (Thy1.1) CD4 or CD8 T cells. (B) Total LN T cell numbers were determined in the mice used in the experiments described in A.*

These results suggested that IL-7/Ab complexes can promote T cell expansion/activation and anti-tumor responses even in non-lymphopenic mice, without producing the enhancing effect on metastasis susceptibility observed in irradiated mice undergoing HTP. To examine this possibility, similar experiments were repeated using IL-7/Ab, IL-2/Ab or both immune complexes (dual IC). In tumor-free mice, these cytokine IC effectively induced T cell proliferation (**Fig 10A**) and accumulation of CD4 and CD8 T cells in both LN and spleen (**Fig 10B**). IL-2/Ab was more effective on CD8 T cells, while IL-7/Ab promoted proliferation of both subsets and was more efficient than IL-2/Ab in inducing T cell accumulation, which is consistent with the known pro-survival activity of IL-7. In addition, IL-7/Ab induced a >5-fold expansion of immature B cells and >2-fold expansion of mature B cells, while both complexes promoted a >2-fold increase in NK cell numbers (not shown). Treatment with these IC was also associated with considerable changes in the phenotype of the expanded T cells. Thus, in mice treated with IL-7/Ab, CD8 T cells exhibited upregulation of CD122 and a transient downregulation of CD127 (at day 3), whereas in mice treated with IL-2/Ab, CD8 T cells showed upregulation of CD122, but no changes in the expression of CD127 (not shown).

To determine whether these cytokine IC could activate T cell effector functions, experiments were performed with OT-1 T cells expressing an ovalbumin (OVA)-specific transgenic T cell receptor (TCR). B6 mice were transfused with 20×10^6 OT-1 CD8 T cells and treated with PBS, IL-7/Ab, IL-2/Ab or both IC. After 7 days, mice were injected with a mixture (1:1) of syngeneic target splenocytes that were either pulsed with $OVA_{257-264}$ peptide (SIINFEKL) and labeled with high doses of CFSE, or non-pulsed and labeled with low doses of CFSE. As shown in **Fig 11**, analysis 4 hr later showed that naïve OT-1 T cells (in PBS-treated recipients) only killed ~25% of the OVA-pulsed targets. In contrast, the

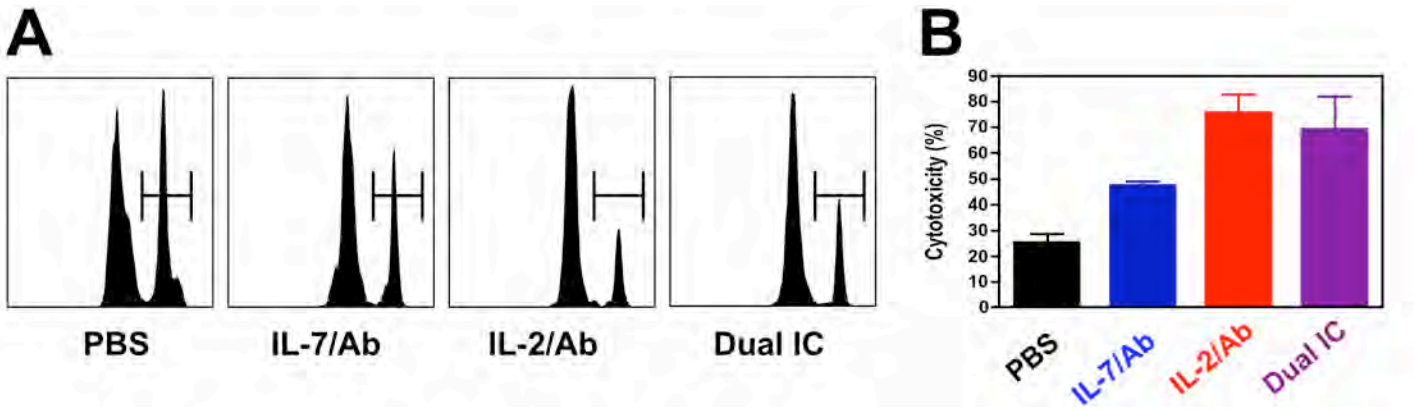


Figure 11. Cytokine/Ab complexes activate cytolytic T cell effector functions in vivo. *(A) B6 mice (CD45.1, Thy1.2) were transfused with 20 x 10^6 OT-1 T cells (CD45.2, Thy1.2) expressing an OVA-specific transgenic T cell receptor, then treated with PBS, IL-7/Ab, IL-2/Ab or both complexes (dual IC) every 3 days. On day 10, mice were injected with a mixture (1:1) of syngeneic splenocytes (CD45.2, Thy1.1) that were either pulsed with OVA-peptide and labeled with high doses of CFSE, or non-pulsed and labeled with low doses of CFSE. Four hours later mice were sacrificed and CFSE profiles on gated Thy1.1 target cells were analyzed by FACS. (B) Percent of cytotoxicity calculated from data generated as described in A using the formula* x = 100 × (50 - "fraction of CFSEhi cells")/50

cytotoxic response was enhanced to ~50% by IL-7/Ab, and to >70% by IL-2/Ab treatment.

Finally, we examined the effect of cytokine IC on 4T1 tumor and metastasis progression. While all IC combinations efficiently extended survival, IL-2/Ab appeared the most efficient in reducing growth of the primary tumor, and establishment of lung metastasis (**Fig 12**). A second experiment using half the amounts of cytokine and antibodies gave virtually identical results (not shown). Overall, these data indicate that T cell trophic cytokines complexed with specific antibodies, particularly IL-2/Ab, effectively induce CD8 T cell expansion, activation of effector functions, and anti-tumor responses, reducing tumor/metastasis and mortality in an aggressive model of breast carcinoma.

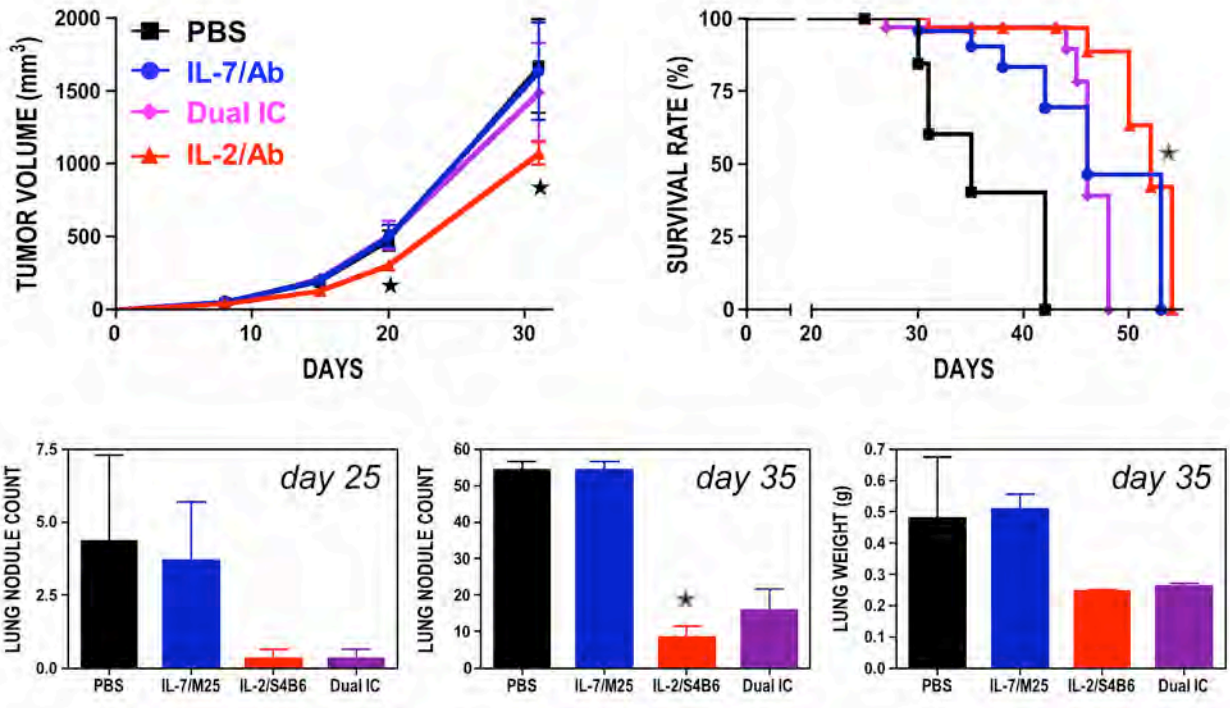


Figure 12. Cytokine/antibody complexes mediate anti-tumor responses in non-lymphopenic mice. *BALB/c mice challenged with 4T1 cells were treated every 3 days with PBS, IL-7/Ab, IL-2/Ab or both complexes, using for each complex 2 μg cytokine and 10 μg Ab. * , $p<0.05$ in t-test.*

Task 3.d. Evaluate whether dendritic cells pulsed with tumor-lysates enhance the anti-tumor response in the ectopic model (accomplished in years 2 and 3)

In the original application, we proposed to use tumor-lysates to stimulate dendritic cells (DC) before injection into tumor-challenged mice. However, a more efficient stimulation may be achieved using apoptotic or necrotic tumor cells in themselves. Indeed, cell apoptosis and necrosis occur in a variety of physiologic and pathologic conditions, including in response to infection and tumor growth. Thus, sensing dying cells, may be an important mechanism to alert the organism, a concept previously proposed as the "danger hypothesis" (33). Consistent with this idea, apoptotic and necrotic cells have been shown to promote strong adjuvant effects (34-36). Although the mechanisms mediating these adjuvant effects are unknown, type I interferons (IFN) produced by DCs in response to apoptotic/necrotic cell products are likely candidates. Considered the most pleiotropic among cytokines, type I IFNs encompass a large family of molecules, of which IFN-α and IFN-β (collectively referred to as IFN-α/β) are the most immunologically relevant. IFN-α/β exert multiple effects on the immune system, including activation of DCs, T cells, B cells and NK cells (37, 38). Importantly, as we have recently reviewed IFN-α/β induced by Toll-like receptor (TLR)-dependent or TLR-independent mechanisms are emerging as being major effectors in autoimmunity (39, 40). Thus, as anti-tumor responses are essentially autoimmune in nature, we have suggested that induction of these cytokines may be critical for effective cancer immunotherapy.

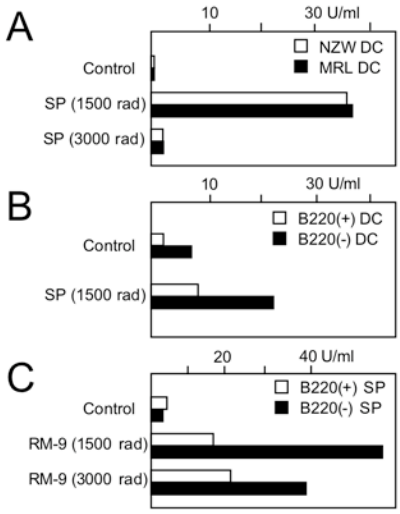


Figure 13. RM-9 carcinoma cells induce production of IFN-α/β by B220(-) DCs. *(A) Flt3L-DCs from NZW and MRL mice were stimulated in vitro with syngeneic irradiated spleen cells (SP). (B) Flt3L-DC from B6 mice were sorted into B220(+) and B220(-) cells and stimulated with syngeneic irradiated spleen cells. (C) Spleen cells from B6 mice were sorted into B220(+) and B220(-) cells and stimulated with irradiated RM-9 cells. IFN-α/β production was tested after 48 hrs incubation by a bioassay with a cell line expressing an ISRE-responsive element luciferase reporter construct.*

To determine whether apoptotic or necrotic cells can activate DCs, induce IFN-α/β, and promote tumor antigen cross-presentation we initially performed a series of *in vitro* and *in vivo* experiments. Splenocytes from NZW and MRL mice were treated with various doses of γ-irradiation, i.e. 1500 rad to induce early apoptosis, 3000 rad to induce advanced apoptosis/necrosis, or 0 rad as control. NZW and MRL mice are autoimmune strains available in our laboratory and were used in this experiment, although similar results were obtained in additional experiments with normal BALB/c and C57BL/6 (B6) mice. Early apoptotic, necrotic and control cells (10^6) were then used to stimulate *in vitro* syngeneic DCs (2.5×10^5) previously derived from bone marrow by stimulation with Flt3L (Flt3L-DCs) as described (41). Flow cytometry analysis confirmed that Flt3L-DCs contained plasmacytoid DC (pDC) subsets ($CD11c^+$ $B220^+$ $PDCA\text{-}1^+$) and conventional $CD11c^+$ $B220^-$ DC subsets (both $CD24^+CD11b^-$ and $CD24^-CD11b^+$) in the expected proportions (41, 42). In addition, as previously reported, Flt3L-DCs showed optimal responses to a variety of stimuli, including polyIC (a stimulus for TLR3), polyU complexed with the liposome DOTAP (TLR7) and CpG ODN-2216 (TLR9) (data not shown). As shown in **Fig 13A**, splenocytes at early apoptotic stages (1500 rad), but not at advanced stages (3000 rad), induced IFN-α/β production by Flt3L-DCs. In further experiments, it was shown that sorted $B220^-$ Flt2L-DCs (containing conventional DCs), but not $B220^+$ Flt3L-DCs (containing pDCs),

produced IFN-α/β in response to apoptotic cells (**Fig 13B**). To determine whether apoptotic tumor cells

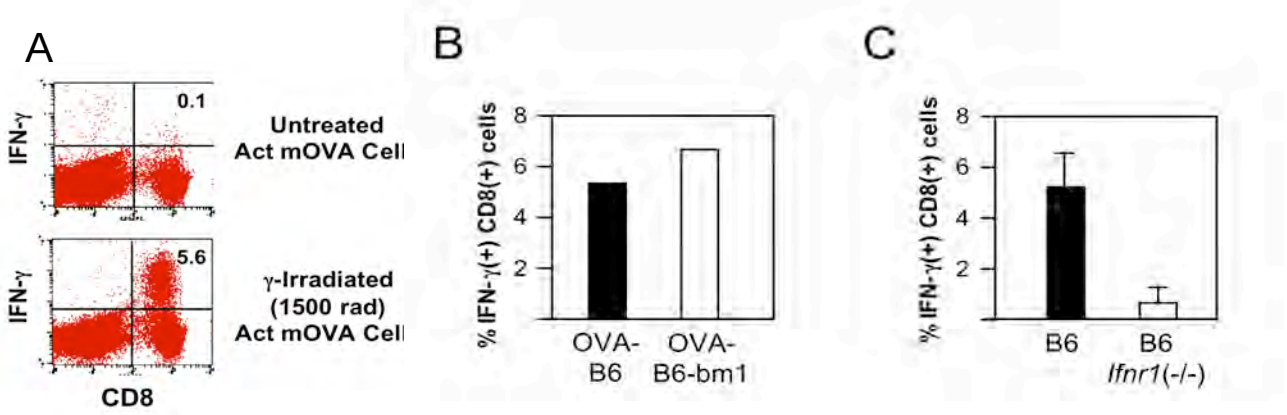


Figure 14. Apoptotic cells expressing membrane OVA induce strong CTL responses in vivo, via IFN-α/β-dependent cross-priming. *(A) Spleen cells from OVA-transgenic mice were irradiated (or not) and injected s.c. in B6 mice. After 8 days, spleen cells were harvested and restimulated in vitro for 6 days. The frequency of antigen specific T cells was then determined by FACS by assessing IFN-γ producing cells in response to $OVA_{257-264}$ peptide. (B) B6 mice were injected with apoptotic cells from either OVA-B6 mice or OVA-B6-bm1 mice (unable to present $OVA_{257-264}$) and analyzed as in A. (C) B6 or IFNAR1-deficient mice were injected with apoptotic splenocytes from OVA-transgenic mice and analyzed as in A.*

can also induce IFN-α/β, RM-9 carcinoma cells were used to stimulate syngeneic splenocytes. As shown in **Fig 13C**, RM-9 cells at early apoptosis stages, but less at advanced (necrotic) stages, induced IFN-α/β production. In particular, response to RM-9 cells was shown by $B220^-$ splenocytes, containing conventional DCs.

To test whether apoptotic cells can prime cytotoxic T cell immune responses *in vivo*, B6 mice were injected s.c. with 10×10^6 cells that were γ-irradiated (1500 rad) or not (control). The injected cells were derived from B6 mice expressing membrane ovalbumin (OVA) as a model antigen. After 8 days, splenocytes were obtained and restimulated *in vitro* for 6 days. The frequency of antigen-specific CD8 T cells was then examined by measuring intracellular IFN-γ production in response to the $H\text{-}2K^b$-binding $OVA_{257-264}$ peptide (SIINFEKL, 5 μg/ml) as described (43). As shown in **Fig 14**, strong CD8 T cell responses were induced only in mice injected with apoptotic (γ-irradiated) cells. To verify that this response is due to cross-priming by host antigen-presenting cells, the experiment was repeated using apoptotic cells from OVA-transgenic B6 mice expressing mutant $H\text{-}2K^{bm1}$, which is unable to present the dominant $OVA_{257-264}$ peptide (44), thereby preventing direct presentation by apoptotic cells. Effective activation of $H\text{-}2K^b$–restricted T cells was observed, confirming that T cell activation occurs *via* cross-priming (**Fig 14B**).

To determine whether T cell cross-priming is IFN-α/β-dependent, WT and IFNAR1-deficient mice lacking the common receptor for IFN-α/β were injected with OVA-transgenic spleen cells and the frequency of IFN-γ-expressing CD8 T cells examined. As shown in **Fig 14C**, T cell responses were severely decreased in IFNAR1-deficient mice, showing the importance of IFN-α/β signaling in this process.

To examine whether vaccination with early apoptotic tumor cells can protect from subsequent tumor cell challenges, an experiment was performed with RM-9 prostate carcinoma cells in a model of experimental metastases. B6 mice (4/group) were injected s.c. with 10×10^6 early apoptotic tumor cells, whereas a second group was not injected (control). Six weeks later, both groups were challenged i.v. with 2×10^5 RM-9 cells to establish experimental lung metastasis, as described (45). All control mice died within 3 weeks with massive metastatic nodules in the lungs. In contrast, all vaccinated mice were still alive 3 weeks after challenge and showed reduced numbers and volumes of lung metastatic nodules

(Fig 15). A similar experiment was repeated with another tumor cell line, the B6-derived breast

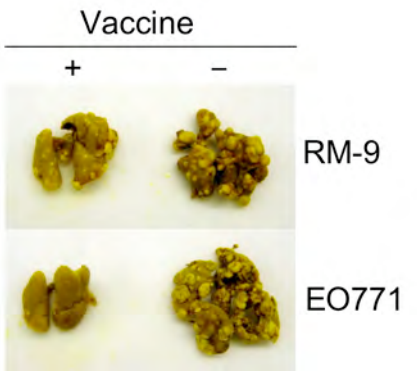


Figure 15. Vaccination with early apoptotic tumor cells protects from induction of experimental lung metastases. *Upper panel, B6 mice were injected (or not, as controls) with 10×10^6 apoptotic tumor cells and, 6 weeks later, challenged i.v. with 2×10^5 RM-9 prostate carcinoma cells. Lungs were examined 3 weeks after challenge. Lower Panel, a similar experiment was performed with EO771 breast carcinoma cells. Lungs were analyzed 4 weeks post-challenge.*

carcinoma EO771 (46). Again, mice were vaccinated s.c. (or not) with 10×10^6 apoptotic tumor cells and, 6 weeks later, challenged i.v. with 2×10^5 EO771 cells. Of the 3 control mice, 2 died within 4 weeks (not examined), while 1 mouse showed enhanced lung metastases and weight (0.660 g) **(Fig 15)**. In contrast, none of the 12 vaccinated mice had died and, upon pathological examination 4 weeks after challenge, all showed dramatically reduced lung nodules and weights (0.200 ± 0.076 g, range 0.118 to 0.399 g) **(Fig 15)**.

Overall, these results suggest that injection of early apoptotic tumor cells, or of B220-negative DCs stimulated *in vitro* with early apoptotic tumor cells, may significantly enhance tumor antigen cross-presentation and priming of specific T cells. Current studies are examining the effect of such manipulations coupled or not with HTP or cytokine/antibody complexes to prevent or treat advanced breast carcinoma and metastasis.

KEY RESEARCH ACCOMPLISHMENTS

- homeostatic T-cell proliferation consistently elicits effective anti-tumor responses, as determined by inhibition of primary tumor growth
- irradiation is more effective than T-cell depletion by antibodies in inducing anti-tumor responses mediated by homeostatic T-cell proliferation
- irradiation (and/or the resulting lymphopenic state) may facilitate metastasis dissemination
- the frequency of T regulatory (Treg) cells increases during homeostatic proliferation, particularly in the presence of a growing breast carcinoma
- in vivo depletion of Treg cells enhances the anti-tumor effect of homeostatic T-cell proliferation on subcutaneous breast carcinoma
- gamma/delta T cells, a lymphocyte subpopulation with significant anti-tumor activity, can be induced to undergo homeostatic proliferation, and this requires depletion of both alpha/beta and gamma/delta T cell compartments and availability of either IL-7 or IL-15
- the anti-tumor response is diminished in aged mice, and this correlates with inefficient homeostatic T-cell proliferation
- homeostatic T-cell proliferation in aged mice can be restored by provision of the T cell trophic cytokine IL-7
- IL-7 complexed with anti-IL-7 antibodies and/or IL-2 complexed with anti-IL-2 antibodies induce T cell proliferation in both lymphopenic and non-lymphopenic mice
- in non-lymphopenic mice, IL-7/antibody and particularly IL-2/antibody complexes induce cytotoxic effector functions in CD8 T cells, and inhibit tumor growth, metastasis and mortality in a model of breast carcinoma
- tumor cells at early apoptotic stages induce production of type I interferons by dendritic cell subsets, promote interferon-dependent cross-priming of specific T cells, and protect mice from subsequent tumor challenges if used as a vaccine

REPORTABLE OUTCOMES

Baccala, R., R. Gonzalez-Quintial, W. Dummer, and A.N. Theofilopoulos. 2005. Tumor immunity via homeostatic T cell proliferation: mechanistic aspects and clinical perspectives. *Springer Semin Immunopathol*. PMID: 15666151

Baccala, R., D. Witherden, R. Gonzalez-Quintial, W. Dummer, C.D. Surh, W.L. Havran, and A.N. Theofilopoulos. 2005. Gamma delta T cell homeostasis is controlled by IL-7 and IL-15 together with subset-specific factors. *J Immunol* 174:4606-4612. PMID: 15814683

Baccala, R., and Theofilopoulos, A.N. 2005. The new paradigm of T-cell homeostatic proliferation-induced autoimmunity. *Trends Immunol* 26:5-8.

Baccala, R., Kono, D.H., and Theofilopoulos, A.N. 2005. Interferons as pathogenic effectors in autoimmunity. *Immunol Rev* 204:9-26.

Baccala, R., Hoebe, K., Kono, D.H., Beutler, B., and Theofilopoulos, A.N. 2007. TLR-dependent and TLR-independent pathways of type I interferon induction in systemic autoimmunity. *Nat Med* 13:543-551.

CONCLUSIONS

Because of the exquisite specificity and effectiveness of the immune system, immunotherapy is a very attractive approach for the treatment of cancer. There is, indeed, a large body of evidence indicating that, if efficiently activated, the immune system can specifically recognize and destroy syngeneic tumor cells of diverse histological origins. However, despite the advances made in recent years in identifying antigenic determinants displayed by cancer cells and devising innovative approaches, clinical success has been limited primarily by the difficulty of overcoming T-cell tolerance for tumor antigens. Based on the observation that, during lymphopenia-induced homeostatic proliferation T cells must interact with self-antigens and acquire a semi-activated state, we and others have suggested that T-cell priming through this process might overcome T-cell tolerance against tumor antigens. We have shown that the anti-tumor effect of homeostatic T-cell proliferation can be used to inhibit tumor progression in a model of breast carcinoma, is optimally promoted by irradiation-induced lymphopenia, and can be enhanced by depleting T regulatory cells or by injecting the cytokine IL-7 complexed with anti-IL-7 antibodies. However, we found that irradiation and/or the associated lymphopenic state may actually facilitate metastasis dissemination. We therefore examined whether T-cell proliferation and activation could be promoted by other means than lymphopenia. The results showed that IL-7/antibody and particularly IL-2/antibody complexes can be used in non-lymphopenic mice to promote T-cell proliferation, activate cytolytic functions, and inhibit tumor growth, metastasis and mortality in a model of breast carcinoma. In additional experiments, it was observed that tumor cells at early apoptotic stages induce production of type I interferons by dendritic cell subsets, promote interferon-dependent cross-priming of specific T cells, and protect mice from subsequent tumor challenges if used as a vaccine. Future studies will examine the mechanisms by which homeostatic T cell proliferation, particularly under the effect of enhanced signaling by various cytokines such as IL-2 and type I interferons and their complexes with specific antibodies, may engage tolerant T cells and promote anti-tumor responses.

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